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Production and Characterization of Amylase from *Calvatia gigantea*

D. KEKOS† AND B. J. MACRIS*

Department of Biology, Nuclear Research Center "Demokritos," Aghia Paraskevi, Attiki, Greece

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α-Amylase (EC 3.2.1.1) was excreted by *Calvatia gigantea* in liquid growth media containing different sources of starch. Among the factors affecting enzyme production in shake flasks were the type and the concentration of starch and the nitrogen source supplied. Optimum cultural conditions for maximum enzyme production were: soluble starch concentration, 5%; inoculum size, 3.75×10^5 conidia per ml; 5-day cultivation time at 28 to 30°C. The observed maximum yield of 81.3 U of saccharifying enzyme activity per ml of growth medium was the highest ever reported in the literature for submerged cultures. Partially purified enzyme functioned optimally at pH 4.5 to 5.5 and 53 to 58°C. The activation energy of enzymic hydrolysis of starch in the range of 20 to 40°C was 8,125 cal/mol (ca. 3.41×10^4 J). The apparent K_m value of the enzyme at 25°C was 7.68×10^{-4} g/ml. Some of the properties of the enzyme under investigation were similar to those of α-amylases excreted from molds producing large amounts of the enzyme.

α-Amylases are all α-1,4-glucan 4-glucanohydrolases (EC 3.2.1.1) and hydrolyze starch, glycogen, and related α-1,4-glucans (13). These enzymes are found in animals (saliva, pancreas), plants (malt), bacteria, and molds (13). Among mold species producing high levels of amylase, those of *Aspergillus niger* and *Aspergillus oryzae* have been used for commercial production of the enzyme (2). It has been demonstrated previously that the production of α-amylase depends upon the strain, the composition of the growth medium, and the methods of cultivation employed (8).

Calvatia gigantea is an edible puffball (1), which is reported here for the first time to excrete elevated quantities of α-amylase when cultivated in liquid growth media containing different sources of starch. This fungus has been previously employed for the production of an antitumor substance (3) and microbial protein from wastes (18). Also, the same fungus was reported to grow on both hydrolyzable and condensed tannins as a sole carbon source (M. Galiotou-Panayotou and B. J. Macris, Int. Ferment. Symp. 22:F-13, 1980).

The present work was undertaken to investigate some of the factors affecting α-amylase production by *C. gigantea* and certain characteristics of the enzyme.

† Present address: Department of Chemical Engineering, Technical University of Athens, Athens, Greece.

MATERIALS AND METHODS

Organism. A laboratory strain of *C. gigantea*, originated from the authors' collection, was used. The fungus was maintained on potato-glucose agar.

Growth medium. The mineral medium contained (g/liter): NaCl, 2; KH_2PO_4, 2.5; $MgSO_4$, 1; $CaCO_3$, 5. The latter compound was used to maintain the pH at 6 to 7 during growth. To avoid hydrolysis of starch, the liquid mineral medium, together with the nitrogen sources, was steam sterilized at 121°C for 30 min, whereas the sources of starch were sterilized in a dry oven at 120°C for 40 min. After sterilization, the two groups of nutrients were mixed together. In all experiments except those in which the effect of nitrogen supplementation was studied, $NaNO_3$ and $(NH_4)_2SO_4$ were added at a concentration of 5 g/liter.

Inoculation and fermentation. A certain inoculum size of conidia was transferred from a stock culture in 250-ml Erlenmeyer flasks containing 100 ml of growth medium. The flasks were incubated for certain periods of time at 28 to 30°C on a rotary shaker operating at 180 rpm. At the end of the incubation period, the fungal biomass and the suspended solids were separated by centrifugation at $2,000 \times g$ for 10 min. The clarified supernatant (crude enzyme) was used as a source of the enzyme.

Preparation of partially purified enzyme. The enzyme was precipitated from the clarified growth medium at 4°C by adding ethanol to a final concentration of 70% by volume. The precipitate was separated by centrifugation at $5,000 \times g$ for 10 min at 4°C, dissolved in 0.05 M acetate buffer (pH 5), and dialyzed overnight against a 200-fold volume of deionized water at 4°C. The dialysate was freeze-dried and used for studying the properties of the enzyme.

Analytical methods. Glucose was determined with a glucose-oxidase-chromogen reagent (ISVT Sclavo, Divisione Diagnostici, Siena, Italy).

Starch was estimated polarimetrically (17). A 2.5-g amount of starchy material was added to 25 ml of 1.124% HCl solution and hydrolyzed for 15 min in a boiling water bath. After the addition of 20 to 30 ml of distilled water, the hydrolysate was cooled to 20°C, and 10 ml of a 4% solution of phosphotungstic acid was added. The volume was brought to 100 ml with distilled water, and the optical rotation of the filtrate was determined. Starch content was calculated by the formula: starch (percent) = $10,000 \times (p - p')/A_D \times L \times M$, where p and p' were the values recorded for the sample and blank, respectively, A_D and L were parameters of the instrument, and M was the weight of the sample used.

Tannin was determined by the method of Folin-Denis (5).

Protein was estimated according to the method of Lowry et al. (9).

Enzyme assay. Saccharifying enzyme activity was determined by the method of Bernfeld (4). A 1-ml amount of properly diluted enzyme was incubated for 3 min at 25°C with 1 ml of 1% starch solution. The reaction was stopped by the addition of 2 ml of dinitrosalicylic reagent. The tube containing this mixture was heated for 5 min in a boiling water bath and cooled in running tap water. After the addition of 20 ml of distilled water, the optical density of the solution was determined at 540 nm. Maltose, ranging from 200 to 2,000 μg/ml, was the standard. One unit of amylase activity was defined as the amount of enzyme required to liberate 1 mg of maltose in 3 min at pH 5 and 25°C.

Dextrinizing enzyme activity was measured by the blue value method of Smith and Roe (21). The blue value was defined as $(D - D') \times D$, where D' was the absorbance at 620 nm of the iodine-substrate complex in the presence of enzyme and D was that without enzyme. One unit of amylase activity was defined as the amount of enzyme required to hydrolyze 1 mg of starch per min at pH 5 and 37°C.

Paper chromatography. Ascending paper chromatography was performed for the analysis of the products of enzymatic action. Chromatograms of Whatman no. 4 filter paper (30 by 40 cm) were used, and the solvent system consisted of propanol-ethyl acetate-water (6:1:3) (11). The substrate was 1% soluble starch, and the reaction was carried out at pH 5 and 37°C. Samples were taken at various times and placed 3 cm apart along a line 4 cm from the bottom of the paper. After drying, the chromatograms were developed with the silver nitrate dip procedure of Ough (11).

RESULTS

Effect of growth conditions on enzyme production. To develop relevant information for the production of the enzyme during cultivation of the fungus on certain starch sources, a number of factors affecting growth were examined. Figure 1 shows the effect of cultivation time, inoculum size, and starch concentration on enzyme production. The time course of the enzyme production showed a maximum after 5 days of cultivation, whereas enzyme yields were increased as both the number of conidia used to inoculate the culture and the starch concentration were elevated. However, when the latter factor was increased to above 5%, a highly viscous culture resulted.

Effect of different starch sources on enzyme production. The fungus was cultivated on a number of starch sources, namely, soluble starch, acorns, wheat flour type 55 (contains 60% starch), vita, and cattle feed flour. The latter two sources are by-products of the milling industry and contain 28 and 22% starch, respec-

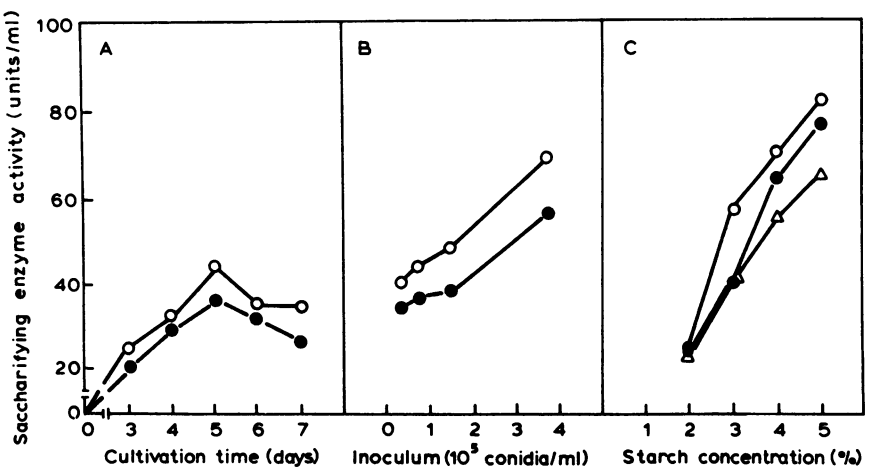

FIG. 1. Effect of cultivation time (A), inoculum size (B), and soluble starch concentration (C) on amylase production. (A) Inoculum size was 0.75×10^5 conidia per ml. Soluble starch concentration was 3% (●) or 4% (○). (B) Cultivation time was 5 days. Soluble starch concentration was 3% (●) or 4% (○). (C) Inoculum size was 3.75×10^5 conidia per ml. Symbols: ●, ○, and △, 4, 5, and 6 days, respectively, as the cultivation time.

tively. The starch content of acorn powder, which was prepared by drying and pulverizing (200 mesh) the perisperm-free seeds of the oak tree, was 60%. The best yield of enzyme was obtained with acorn powder (Fig. 2).

Effect of nitrogen supplementation on enzyme production. A number of inorganic and organic nitrogen sources were examined to determine their effects on enzyme production. Both inorganic and organic nitrogen markedly enhanced enzyme production (Fig. 3). The best results with inorganic nitrogen were obtained when $(NH_4)_2SO_4$ was added to growth media containing 4% soluble starch. In the same media, the best enzyme yields were obtained with casein supplementation.

Enzyme production in the presence of tannins. Figure 4 shows the results of growing *C. gigantea* in the presence of tannins (3% acorn medium) for α-amylase production. The acorn medium contained about 1.1 mg of tannins per ml, resulting from the extraction of acorn. From these data it is evident that the presence of tannins in the growth medium did not seem to affect enzyme excretion. On the contrary, the observed yields with acorn were higher than those obtained with 3% soluble starch. On the other hand, the total tannin content of the growth medium was reduced (possibly by biodegradation) during fermentation to about one-

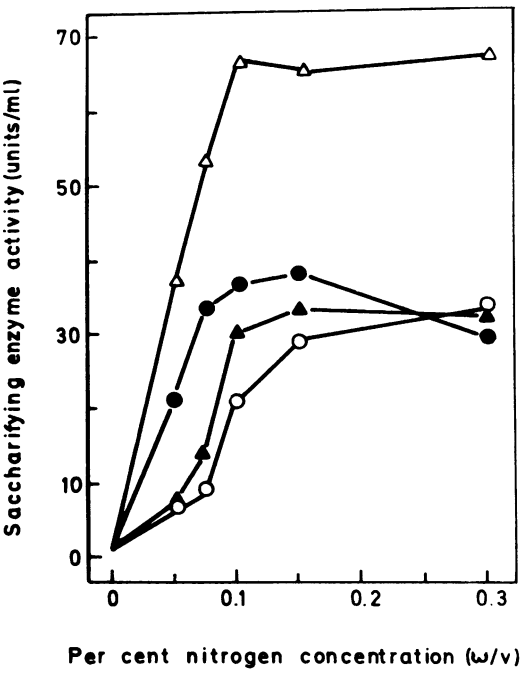

FIG. 3. Effect of nitrogen supplementation of the growth medium (4% soluble starch) on amylase production. Nitrogen concentration was expressed as elemental nitrogen. Inoculum size was 10^5 conidia per ml. Cultivation time was 5 days. Symbols: ○, $NaNO_3$; ●, $(NH_4)_2SO_4$; ▲, urea; △, casein.

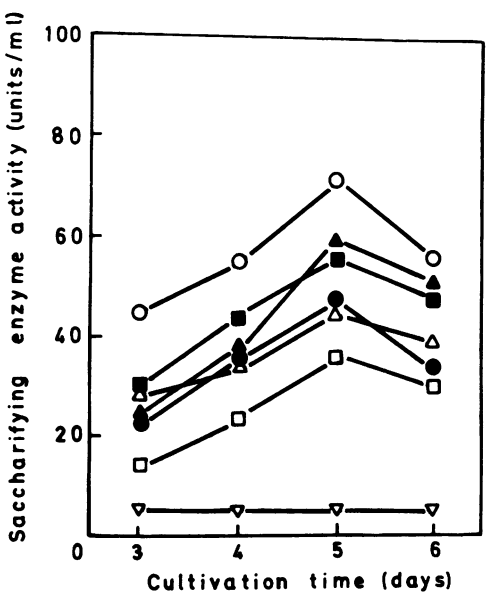

FIG. 2. Effect of starch source on amylase production. Inoculum size was 1.5 × 10^5 conidia per ml. Cultivation time was 5 days. Symbols: ▽, ●, ■, soluble starch concentration of 1, 3, and 5%, respectively; □, 5% cattle feed; ▲, 5% wheat flour; ○, 5% acorn.

fourth of the original concentration.

Partial purification of the enzyme. The enzyme was partially purified by ethanol precipitation and dialyzed as described above. A two-fold increase in the specific activity of the enzyme was obtained (Table 1).

Properties of the enzyme. (i) Effect of pH. The effect of pH on enzyme activity is shown in Fig. 5. Both crude and partially purified enzymes were tested in the pH range 3.5 to 5.5 in 0.05 M acetate buffer and in the pH range 5.5 to 7.5 in 0.05 M phosphate buffer at 25°C. Optimum enzyme activity was observed at pH 4.5 to 5.5, and 50% of the maximum activity was lost at pH 7.2.

(ii) Effect of temperature. The optimum effect of temperature on enzyme activity in 0.05 M acetate buffer (pH 5) was observed between 53 and 58°C (Fig. 6). An Arrhenius plot (the logarithm of reaction rate versus the reciprocal of the absolute temperature) allowed the calculation of an average energy of activation for the enzymic hydrolysis of starch equal to 8,125 cal/mol (ca. 3.41 × 10^4 J) in the range of 20 to 40°C.

(iii) Effect of substrate concentration. The effect of substrate on enzyme activity was determined in 0.05 M acetate buffer (pH 5) at 25°C.

TABLE 1. Flow sheet for purification of *C. gigantea* amylase

Fraction	Vol (ml)	Activity[a]	Sp act[b]	Total activity	Yield (%)	Purification factor
Crude	100	45.8	89.8	45.8	100.0	1.00
Ethanol precipitation	100	40.6	147.0	40.6	88.7	1.64
Dialysis	100	35.4	182.5	35.4	72.7	2.03

[a] Saccharifying activity expressed in units per milliliter of culture medium.
[b] Saccharifying activity expressed in units per milligram of protein.

The Lineweaver-Burk plot allowed the calculation of a K_m equal to 7.68×10^{-4} g/ml at 25°C.

(iv) Effect of chemicals. A number of chemicals were tested for their effect on enzyme activity. The tests were carried out in 0.05 M acetate buffer (pH 5) at 25°C (Table 2). From the results it was evident that Cu^{2+} and Hg^{2+} were inhibitory to the enzyme. This inhibition was particularly reversed by EDTA and, in the case of Cu^{2+}, by histidine. The latter amino acid and Fe^{2+} enhanced significantly enzyme activity, whereas the presence of K^+ and Na^+ ions had no effect on the enzyme.

Determination of type of amylase. The type of amylase was determined by the method of Robyt and French (15) using the blue value-reducing value curve for the amylase from *C. gigantea* and from a commercial preparation of *A. niger* α-amylase (Sigma Chemical Co., α-amylase, type IV-A) (Fig. 7). The curve for the *C. gigantea* enzyme was similar to that of *A. niger* α-amylase.

Paper chromatography. The pattern of action of the enzyme was examined on soluble starch (Fig. 8). The products of the digestion included large starch oligomers at the early stages of the reaction. As the reaction proceeded, the digestion products included maltose and, to a lesser extent, glucose.

DISCUSSION

The present work reports for the first time on the production of amylase by *C. gigantea*. More important than this is the fact that the fungus under investigation was found to be among the fungi reported in the literature as producing the highest levels of amylase.

The data on the effect of cultivation time, inoculum size, and substrate concentration (Fig. 1) show an optimum of 5 days for cultivation

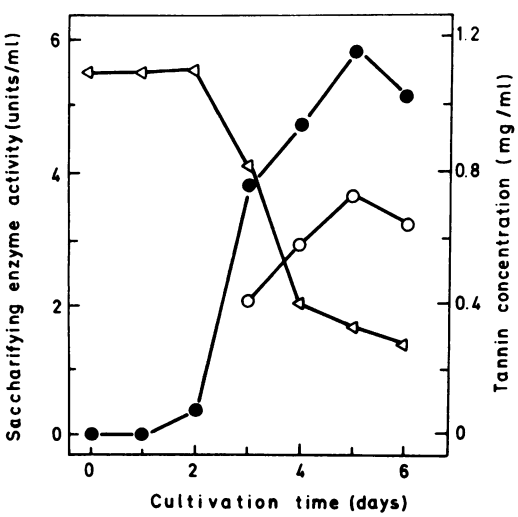

FIG. 4. Amylase production in the presence of tannins. Inoculum size was 10^5 conidia per ml. Cultivation time was 5 days. Symbols: ○, amylase activity produced on 3% soluble starch; ●, amylase activity produced on 3% acorn medium; ▽, tannins.

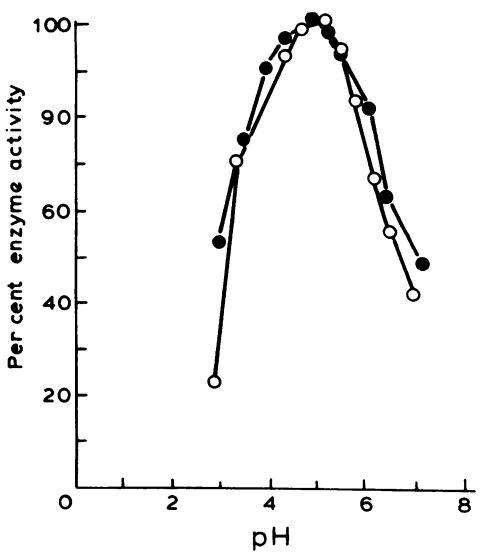

FIG. 5. Effect of pH on amylase activity measured as saccharifying activity. Symbols: ●, crude enzyme; ○, purified enzyme.

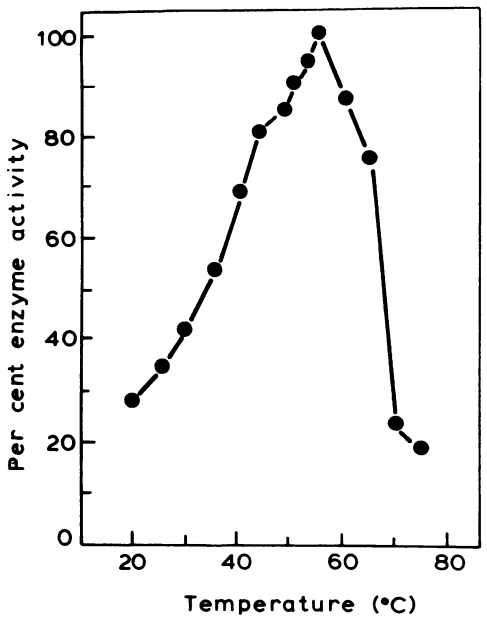

FIG. 6. Effect of temperature on amylase activity measured as saccharifying activity.

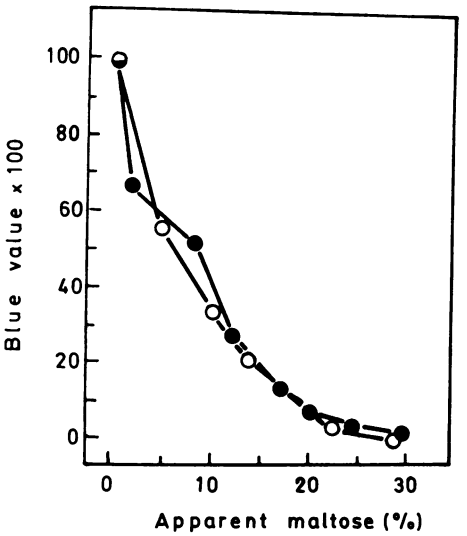

FIG. 7. Blue value-reducing value curves of the amylase from *C. gigantea* and *A. niger* α-amylase. Enzyme activity (0.2 U of saccharifying activity per ml) was determined at pH 5 (0.05 M acetate buffer) and 32°C. Symbols: ●, *C. gigantea* amylase; ○, *A. niger* amylase.

time and an increase of the enzyme yield as both inoculum size and starch concentration were increased. The optimum cultivation time reported for *A. niger* (19) and *A. oryzae* (8) grown in a starch liquid medium was 144 and 72 h, respectively.

When the fungus was cultivated on different sources of starch, the best results were obtained with acorns. Acorn medium contains relatively high amounts of tannins, the presence of which did not seem to inhibit either growth of the fungus or enzyme excretion, although tannins are well-known inhibitors of microbial growth and enzyme activity (7, 12, 20). A strong inhibitory effect of tannins on certain bacterial species was reported at tannin concentrations of about 40 to 100 times lower than those used in the present work (7). The disappearance of tannins during growth could be explained on the basis of

TABLE 2. Effect of chemicals on amylase activity

Treatment[a]	Relative enzyme activity (%)		
	Chemical alone	With EDTA[a]	With histidine[a]
Control	100	98	110
Fe^{2+}	123	101	125
Ca^{2+}	95	98	120
Cu^{2+}	24	76	62
Hg^{2+}[b]	20	55	
Zn^{2+}	93	93	102
Control	100		
KCl	103		
KBr	100		
KI	105		
NaCl	102		

[a] Ion concentration was 10^{-2} M.
[b] Concentration was 10^{-4} M.

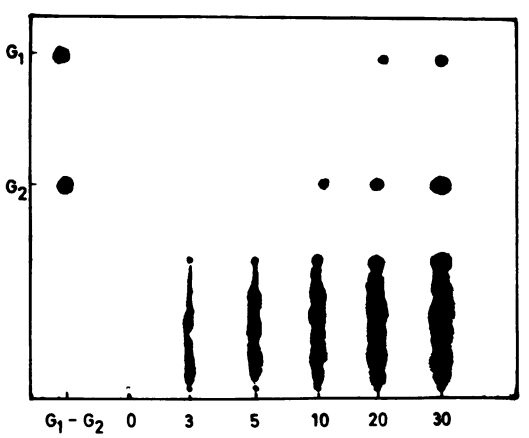

FIG. 8. Chromatographic analysis of soluble starch digestion by the amylase from *C. gigantea*. The reaction contained 10 mg of soluble starch and 0.2 U of saccharifying activity per ml. After incubation at 37°C for various time intervals, 10-μl portions were applied on the chromatogram. G_1, Glucose; G_2, maltose.

TABLE 3. Comparison of α-amylases produced by *C. gigantea* and certain other molds producing high levels of the enzyme

Mold	Substrate (carbon source)	Maximum enzyme yield[a]		Enzyme properties			Effect of chemicals	Reference
		Dextrinizing activity	Saccharifying activity	Optimum pH	Optimum temp (°C)	K_m		
A. niger PRL 558	8% soluble starch	85 (30°C)		4.5–5.0	60			19
A. oryzae	2% soluble starch	17 (30°C)		5.5–5.9	40			—[b]
A. oryzae EI 212	2% soluble starch		40 (30°C)	4.5–5.0	50–55	3.85×10^{-3}	Inhibition by Ca^{2+}. No effect with Cl^-.	8
C. gigantea	5% acorn		71 (25°C)	4.5–5.5	53–58	7.68×10^{-4}	Inhibition with Cu^{2+} and Hg^{2+}. No effect with K^+ or Na^+.	
C. gigantea	5% soluble starch		81.3 (25°C)	4.5–5.5	53–58	7.68×10^{-4}	Inhibition with Cu^{2+} and Hg^{2+}. No effect with K^+ or Na^+.	
C. gigantea	5% soluble starch	148 (30°C)		4.5–5.5	53–58	7.68×10^{-4}		

[a] Expressed as units per milliliter of growth medium. The temperature at which the enzyme activity was assayed is given in parentheses.

[b] —, V. R. Feniskova, Proc. Intern. Symp. Enz. Chem., Tokyo and Kyoto, Japan, p. 482–485, 1957.

the known ability of *C. gigantea* to utilize tannins as a sole carbon source (M. Galiotou-Panayotou and B. J. Macris, Int. Ferment. Symp. 22:F-13, 1980).

Nitrogen supplementation with inorganic and organic sources markedly affected enzyme production. The best results were obtained with organic nitrogen, and similar results were reported elsewhere (19).

The characteristic blue value-reducing value curve of the amylase excreted by *C. gigantea* was similar to that of α-amylase from *A. niger* and identified the enzyme as being an alpha-type amylase (Fig. 7). McWethy and Hartman (10) reported similar patterns of curves for α-amylases excreted by *Bacillus subtilis, Bacteroides amylophilus*, and *A. oryzae*, whereas the pattern of the curve of barley β-amylase was quite different.

The degradation products of soluble starches produced by the amylase of *C. gigantea* (Fig. 8) were similar to those of many other α-amylases previously examined (6, 10, 14, 16, 22). Although the distribution of products varied somewhat from other α-amylase digests, it was evident that, overall, the *C. gigantea* enzyme digests were characteristic of α-amylase action patterns.

The optimum concentration of the α-amylases excreted by *C. gigantea* and by certain other molds producing high levels of the enzyme appear in Table 3. From these data, it is evident that both the optimum concentration of the carbon source and the properties of the enzyme under investigation are within the range reported for other high-amylase molds. Moreover, the yields of α-amylase obtained with *C. gigantea* were higher than those reported for the molds listed in Table 3, even higher than the yield reported for *A. oryzae*, which is a high-amylase mold (8).

In conclusion, the edible puffball, *C. gigantea*, proved to be a potential α-amylase producer when cultivated in a variety of starch sources. In addition to that, the excretion of the enzyme was not inhibited by the presence of tannins, a fact which is of particular importance for the utilization of starch sources containing these highly inhibitory substances.

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